IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of	)	
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Yasuki Kato, et al.	)	
	:	Group Art Unit: 1615
Serial No. 10/018,349	)	
	:	Examiner: Gollamudi S. Kishore, Ph.D
Filed: December 19, 2001	)	
	:	
For: METHOD OF INHIBITING	)	
LEAKAGE OF DRUG	)	
ENCAPSULATED IN	)	
LIPOSOMES	)	

DECLARATION

The Honorable Commissioner of
Patents and Trademarks
Washington, D.C. 20231

Sir:

I, Masahiro Yamauchi of 410-1-A-201, Nameri, Nagaizumi-cho, Sunto-gun, Shizuoka, 411-0933, Japan, do declare as follows:

I finished my master course at Department of Polymer Chemistry, Faculty of Engineering, Kyoto University in March, 1994, and I was given the degree of Master. Since April, 1994, I have been employed by Kyowa Hakko Kogyo Co., Ltd., the assignee of the above-identified application, and have engaged

in the research on drug formulation at Pharmaceutical Research Center of the company.

The following experiment was conducted under my direction.

EXPERIMENT

The liposomes containing UCN-01 of the present invention (Examples 5 to 8) and the liposome to be compared with the present invention (Comparative Examples 6) were prepared and the leakage of UCN-01 in rat plasma was evaluated (Test Example 3). The relationship between the remaining ratio (%) and the particle size of liposomes was plotted in Figure 1 based on the results of Test Examples 1 to 3 in the present invention.

Example 5

To 5 g of hydrogenated soybean phosphatidylcholine (HSPC) was added 25 mL of a 100 mmol/L citrate buffer (pH 4.0), followed by shaking under stirring with a vortex mixer, and the resulting suspension was passed through a polycarbonate membrane filter (4 times through 0.4 μm filter, then 10 times through 0.2 μm filter) at 70°C. Then, a 100 mmol/L citrate buffer was added thereto to give a liposome suspension having a concentration of HSPC of 62.5 mg/mL. To 8 mL of the above liposome suspension was added 10 mg of UCN-01. The pH of the resultant mixture was adjusted to approximately 8 by adding an appropriate amount of 1 mol/L aqueous sodium hydroxide. Then, distilled water was added thereto to give a total volume of 10 mL. The mixture was heated at 70°C for 5 minutes to give a liposome suspension encapsulated UCN-01 (A).

The average particle diameter of the liposomes measured by the DLS method was 150 nm.

Example 6

To 5 mL of the liposome suspension containing UCN-01 (A) as prepared in Example 5 was added 0.05 mL of a 1.25 g/mL solution of PEG-modified distearoyl phosphoethanolamine (PEG-DSPE) in ethanol. Then, the mixture was heated at 70°C for 2 minutes to thereby coat the surface of the liposomes with PEG.

The average particle diameter of the liposomes measured by the DLS

method was 152 nm.

Example 7

To 5 g of HSPC was added 25 mL of a 100 mmol/L citrate buffer (pH 4.0), followed by shaking under stirring with a vortex, and the resulting suspension was passed through a polycarbonate membrane filter (10 times through 0.4 μm filter) at 70°C. Then, a 100 mmol/L citrate buffer was added thereto to give a liposome suspension having a concentration of HSPC of 62.5 mg/mL. To 8 mL of the above liposome suspension was added 10 mg of UCN-01. The pH of the resultant mixture was adjusted to approximately 8 by adding an appropriate amount of 1 mol/L aqueous sodium hydroxide. Then, distilled water was added thereto to give a total volume of 10 mL. The mixture was heated at 70°C for 5 minutes to give a liposome suspension encapsulated UCN-01 (B).

The average particle diameter of the liposomes measured by the DLS method was 216 nm.

Example 8

To 5 mL of the liposome suspension containing UCN-01 (B) as prepared in Example 7 was added 0.05 mL of a 1.25 g/mL solution of PEG-DSPE in ethanol. Then, the mixture was heated at 70°C for 2 minutes to thereby coat the surface of the liposomes with PEG.

The average particle diameter of the liposomes measured by the DLS method was 213 nm.

Comparative example 6

To 2 g of HSPC was added 10 mL of a 100 mmol/L citrate buffer (pH 4.0), followed by shaking under stirring with a vortex mixer, and the resulting suspension was passed through a polycarbonate membrane filter (4 times through 0.4 μm filter, 4 times through 0.1 μm filter and then 10 times through 0.08 μm) at 70°C. Then, a 100 mmol/L citrate buffer was added thereto to give a liposome suspension having a concentration of HSPC of 62.5 mg/mL. To 4 mL of the above liposome suspension was added 5 mg of UCN-01. The pH of the resultant mixture was adjusted to approximately 8 by adding an appropriate amount of 1 mol/L aqueous sodium hydroxide. Then, distilled water was added thereto to give a total volume of 5 mL. The mixture was heated at 70°C for 5 minutes to give a liposome suspension encapsulated UCN-01 (C).

The average particle diameter of the liposomes measured by the DLS method was 98 nm.

Test Example 3

The leakage of UCN-01 encapsulated in liposomes prepared in Examples 5-8 and Comparative Examples 6 was monitored in the same manner as in Test Example 1 of the present invention.

The results were shown in Table 3.

Table 3

		UCN-01 remaining ratio (%)
Example 5	Immediately after mixing	98
	After 3 hours	81
Example 6	Immediately after mixing	87
	After 3 hours	73
Example 7	Immediately after mixing	99
	After 3 hours	88
Example 8	Immediately after mixing	91
	After 3 hours	82
Comparative Example 6	Immediately after mixing	94
	After 3 hours	25

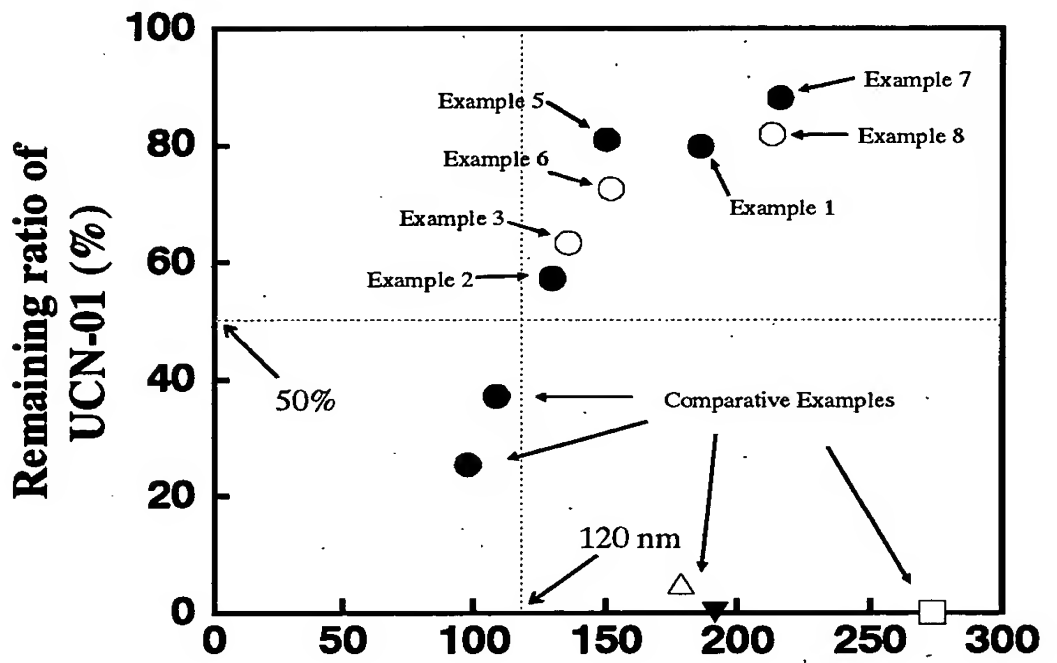
The summary of all results of the Test Example according to the present invention was shown in Table 4. The results of Test Example 1 was referred to p.16 in the specification, that of Test Example 2 was referred to PETITION FOR EXTENSION OF TIME AND AMENDMENT dated November 20,2003, and that of Test Example 3 was referred to this Declaration.

Table 4 Summary of the results of Test Examples 1 to 3

		particle size (nm)		UCN-01 remaining ratio (%)
Test Example 1	Example 1	186	Immediately after mixing	95
			After 3 hours	80
	Example 2	130	Immediately after mixing	91
			After 3 hours	57
	Example 3	136	Immediately after mixing	94
			After 3 hours	63
Test Example 3	Example 5	150	Immediately after mixing	98
			After 3 hours	81
	Example 6	152	Immediately after mixing	87
			After 3 hours	73
	Example 7	216	Immediately after mixing	99
			After 3 hours	88
	Example 8	213	Immediately after mixing	91
			After 3 hours	82
Test Example 1	Comparative Example 1	109	Immediately after mixing	90
			After 3 hours	37
	Comparative Example 2	274	Immediately after mixing	23
			After 3 hours	0
	Comparative Example 3	179	Immediately after mixing	93
			After 3 hours	5
Test Example 2	Comparative Example 4	192	Immediately after mixing	86
			After 3 hours	0
Test Example 3	Comparative Example 6	98	Immediately after mixing	94
			After 3 hours	25

Further, in Figure 1, the relationship between the remaining ratio (%) and the particle size of liposomes was plotted based on Table 4.

Figure 1 Relationship between the remaining ratio (%) of UCN-01 encapsulated in liposomes and the particle size of liposomes in rat plasma containing human AGP after 3 hours at 37℃



●: hydrogenated soybean phosphatidylcholine (HSPC) liposomes of Example 1, 2, 5 and 7, and Comparative Example 1 and 6
○: mixed lipids of HSPC and PEG-modified distearoyl phosphoethnolamine (PEG-DSPE) liposomes of Example 3, 6 and 8
△: dipalmitoyl phosphatidylcholine (DPPC) liposomes of Comparative Example 3
▼: HSPC/PEG-DSPE/Cholesterol liposomes of Comparative Example 4
□: Egg phosphatidylcholine (Egg PC) liposomes of Comparative Example 2

Conclusion

As shown in Figure 1, the liposomes made from HSPC showed the enhanced remaining ratio of UCN-01 in the liposomes depending on the particle size, that is the inhibition of leakage of UCN-01 depending on the particle size. The inhibition ratio of more than 50% was observed on the liposomes having the particle size of more than 120 nm. The liposomes made from the mixed lipids of HSPC and PEG-modified phospholipid showed the same nature as above. The liposomes made from other lipids (for example, Egg phosphatidylcholine (Egg PC), dipalmitoyl phosphatidylcholine (DPPC), mixed lipids of HSPC, PEG- DSPE and cholesterol) did not show the inhibition of leakage from the liposomes.

The undersigned declarant declares further that all statements made herein of his knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Executed this day of , 2005.

Masahiro Yamauchi

Masahiro Yamauchi